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INTRASTRIATAL GRAFTS OF EMBRYONIC MESENCEPHALIC RAT NEURONS GENETICALLY MODIFIED USING AN ADENOVIRUS ENCODING HUMAN Cu/Zn SUPEROXIDE DISMUTASE

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Abstract—Intrastriatal grafting of embryonic dopamine-containing neurons is a promising approach for treating clinical and experimental Parkinson's disease. However, neuropathological analyses of grafted patients and transplanted rats have demonstrated that the survival of grafted dopamine neurons is relatively poor. In the present study, we pursued a strategy of transferring a potentially neuroprotective gene into rat embryonic mesencephalic rat cells *in vitro*, before grafting them into the denervated striatum of 6-hydroxydopamine-lesioned rats. We performed intrastriatal grafts of embryonic day 14 mesencephalic cells infected with replication-defective adenoviruses bearing either the human copper-zinc superoxide dismutase gene or, as a control, the *E. coli* lac Z marker gene. The transgenes were expressed in the grafts four days after transplantation and the expression persisted for at least five weeks thereafter. After five weeks postgrafting, there was more extensive functional recovery in the superoxide dismutase group as compared to the control (uninfected cells) and β-galactosidase groups. The functional recovery was significantly correlated with the number of tyrosine hydroxylase-positive cells in the grafts, although the clear trend to increased survival of the dopamine neurons in the superoxide dismutase grafts did not reach statistical significance.

Only a moderate inflammatory reaction was revealed by OX-42 immunostaining in all groups, suggesting that *ex vivo* gene transfer using adenoviral vectors is a promising method for delivering functional proteins into brain grafts. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: neural graft, mesencephalic cells, superoxide dismutase, adenovirus, *ex vivo* gene transfer, Parkinson's disease.

Transplants of dopamine-containing neurons into the brain of patients with Parkinson's disease (PD) have been found to survive and provide marked amelioration of motor symptoms. However, the symptomatic relief is far from complete and one possible contributing factor to the limited effect is poor survival of the implanted neurons.[21,29] It is estimated that only 5–20% of grafted dopamine neurons survive the transplantation procedure.[8,15,24,25,41] Therefore, several studies have focused on developing new strategies for enhancing the viability of embryonic dopamine neurons after transplantation into animals with experimental brain lesions.[22,24,36,42,46,50]

Because dopamine neurons can generate free radicals by autoxidation or monoamine oxidase-mediated metabolism of dopamine,[28] they may be particularly susceptible to damage by oxidative stress. Oxygen free radical production may increase during preparation and implantation of the graft tissue due to cellular hypoxia and trauma, causing further death of grafted dopamine neurons.[24] We recently demonstrated an increased dopamine neuron survival in transplants prepared from ventral mesencephalic (VM) tissue taken from transgenic mice that overexpress Cu/Zn superoxide dismutase (CuZn-SOD),[25] an enzyme which plays a critical role in the detoxification of oxygen free radicals.[14] This study strongly suggested that intracellular overexpression of CuZnSOD is an effective means of protecting dopamine neurons against free radicals generated during the grafting procedure.

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Abbreviations: Ad-hCuZnSOD, recombinant adenovirus encoding the human Cu/Zn SOD; Ad-RSVβgal, recombinant adenovirus encoding the *E. coli* β-galactosidase; βgal, β-galactosidase; CR3, complement receptor 3; DA, dopamine; HBSS, Hank's balanced salt solution; MOI, multiplicity of infection; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; pfu, plaque forming unit; RSV, Rous sarcoma virus; SOD, superoxide dismutase; TH, tyrosine hydroxylase; VM, ventral mesencephalon.

One attractive strategy for producing high levels of bioactive proteins in neurons is the use of replication-defective adenovirus vectors, which allow delivery of transgenes into post-mitotic cells.[19] Thus, an adenovirus encoding CuZnSOD could be an interesting tool when trying to protect grafted dopamine neurons against free radical toxicity. We previously reported the construction of a replication-defective adenovirus containing human CuZnSOD cDNA (Ad-hCuZnSOD)[3]. This recombinant adenovirus efficiently drove the intracellular expression of human CuZnSOD *in vitro*, and was found to protect infected striatal cells in culture against glutamate neurotoxicity.

Reports regarding *ex vivo* or *in vivo* adenovirus-mediated gene transfer have recently focused on potentially detrimental effects of the gene transfer procedure. A marked inflammatory response has been described following *in vivo* injection into the rat striatum of a replication-defective adenovirus either containing or lacking the *E. coli* LacZ gene.[9] Moreover VM tissue infected *ex vivo* with adenoviral vectors encoding either *E. coli* LacZ or human brain-derived neurotrophic factor (BDNF) was reported to survive poorly upon subsequent intrastriatal grafting and presumably this was due to the increased host inflammatory response.[37] Therefore, the main objectives of the present study were to investigate further the feasability of the *ex vivo* adenovirus-mediated gene transfer approach and to examine whether it is possible to develop an effective gene transfer protocol that does not jeopardize graft survival or function.

Embryonic VM cells were genetically modified *ex vivo* to overexpress CuZnSOD using the hCuZnSOD recombinant adenovirus, and were subsequently transplanted into the denervated striatum of adult rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the mesostriatal pathway. We evaluated the survival and the functional efficacy of transplants infected with recombinant adenoviruses encoding either *E. coli* LacZ (Ad-RSVβgal) or human CuZnSOD (Ad-hCuZnSOD). In addition, the inflammatory response to cells subjected to adenoviral gene transfer was monitored by immunohistochemical detection of macrophages and microglia.

EXPERIMENTAL PROCEDURES

Recombinant adenoviral vectors

Recombinant replication-defective adenoviruses bearing the *Escherichia coli* LacZ marker gene (Ad-RSVβgal) or the human copper-zinc superoxide dismutase gene (Ad-hCuZnSOD) were obtained as described previously.[3,45] Briefly, the LacZ gene and the hCuZnSOD cDNA were inserted downstream from the long terminal repeat of the Rous Sarcoma Virus promoter (RSV) in a plasmid (shuttle vector) containing the inverted terminal repeat of the adenoviral genome, encapsidation sequences, and adenoviral sequences allowing homologous recombination with the right part of the viral genome. After their linearization, the shuttle vector and the large ClaI fragment of the type 5-adenovirus DNA were co-transfected into the transformed human kidney cell line 293 using the calcium phosphate-DNA precipitation method. The transfected cells were overlaid with agar, and plaques were screened for the presence of the recombinant adenovirus. Viral stocks were prepared by expansion and purification of the recombinant adenoviruses. Virus titres were determined by plaque assays on 293 cells and expressed as plaque forming units (pfu) /ml. Ad-hCuZnSOD and Ad-RSVβgal were obtained at titres of 3×10^{10} and 10^{11} pfu/ml, respectively.

Lesion surgery and turning behaviour

Unilateral lesion of the ascending mesostriatal dopamine pathway of adult female Sprague–Dawley rats (bred under Special Pathogen Free (SPF) conditions, B & K Universal, Sollentuna, Sweden) was achieved by stereotaxic injection of 6-OHDA (Sigma, Sweden) into the medial forebrain bundle as described previously.[15] The rats were tested for amphetamine-induced (2.5 mg/kg, i.p.) turning behaviour.[48] The rotational behaviour was monitored in automated rotameters for 90 min. A net rotational asymmetry score was calculated by subtracting the number of turns contralateral to the lesion from the number of ipsilateral turns. For transplantation surgery, we selected the rats that exhibited a net rotation score of at least six full turns/min towards the lesioned side, which is consistent with a near complete unilateral depletion of dopamine.[44] At two and five weeks after transplantation, the rats were tested again for rotational asymmetry using the same protocol.

Preparation and infection of embryonic ventral mesencephalon cells

Cell suspensions of VM tissue were obtained from embryonic day 14 rats derived from three pregnant Sprague–Dawley females. The embryonic brains were dissected in Hank's balanced salt solution (HBSS, Gibco, Sweden) and the pieces of VM tissue obtained were incubated in HBSS containing 0.1% trypsin (Worthington, USA)/0.05% DNase (Sigma, USA) at 37°C for 20 min. After repeated rinsing with HBSS/0.05% DNase, the tissue was mechanically dissociated into a single-cell suspension with fire-polished Pasteur pipettes. We prepared a total of three cell suspensions, each containing tissue from 12 embryos dissociated in 80 ml of medium. Two cell suspensions were then infected with either hCuZnSOD or RSVβgal recombinant adenoviruses. The viruses were directly added to the cell suspensions at a multiplicity of infection (MOI) of 25 pfu/cell, and the cell suspensions were then immediately incubated at 37°C for 1 h. Each cell suspension was agitated briefly every 15 min to promote mixing of the cells and viruses. A control cell suspension, mock-infected with buffer alone, was incubated under the same conditions. Cell concentration and viability was determined using Trypan Blue dye exclusion. For all three groups, the cell viability was over 90%, and the cell concentrations in the control, βgal and superoxide dismutase (SOD) groups were $3.65 \times 10^4/\mu l$, $4 \times 10^4/\mu l$ and $3.99 \times 10^4/\mu l$, respectively.

Transplantation surgery

One or two months after having 6-OHDA lesion surgery, 24 rats were divided into three groups (n=8 each) and received control uninfected transplants (control group) or transplants infected with either Ad-RSVβgal (βgal group) or Ad-hCuZnSOD (SOD group). Two implants of cells (2 μl each, giving a total of 146,000–160,000 cells/rat) were stereotaxically deposited into the 6-OHDA denervated, right striatum of anaesthetized rats as described previously.[15] The injection coordinates were (with respect to bregma and dura) A: 0.7 mm, L: 2.3 mm and 3.2 mm, V: 4.5 mm, with the tooth bar set at zero.

Tissue preparation

To verify the transgene expression and to monitor the extent of inflammatory response, two grafted rats/group

were perfused four days after transplantation surgery. The remaining rats (n=6 for each group) were perfused five weeks after transplantation surgery. Rats were deeply anaesthetized with chloral hydrate and perfused transcardially with saline followed by a solution of 0.1 M phosphate buffer containing 4% paraformaldehyde, pH 7.4. Brains were removed, postfixed for 4 h in 4% paraformaldehyde, cryoprotected in 20% sucrose overnight, and 30 µm coronal sections were cut on a freezing microtome. Free-floating sections were processed for βgal histochemistry, and for tyrosine hydroxylase (TH), hCuZnSOD, βgal and OX-42 immunohistochemistry (for details see below).

β-galactosidase histochemistry

β-gal activity was detected by incubating the brain sections for 3 h at 37°C in an X-gal solution consisting of 0.1 M phosphate-buffered saline (PBS) with potassium ferricyanide (4 mM, Sigma), potassium ferrocyanide (4 mM, Merck, Germany), $MgCl_2$ (4 mM, Merck) and X-gal (0.4 mg/ml, Appligene, USA).

Immunohistochemistry

After quenching of endogenous peroxidase with 3% H_2O_2 in 0.1 M PBS, and blocking of nonspecific staining with 10% serum in 0.3% Triton X-100, brain sections were incubated overnight with primary antibodies against hCuZnSOD (Valbiotech, France, 1:500), β-gal (Cappel, USA, 1:1000), TH (Pel-Freez, USA, 1:500) or OX-42 (Serotec, UK, 1:300) at room temperature. Sections were then incubated with the following biotinylated secondary antibodies: anti-sheep/goat Ig (Amersham, UK), anti-rabbit Ig (Vector, USA), anti-rabbit Ig (Vector) and anti-mouse Ig (Vector) for hCuZnSOD, βgal, TH and OX-42, respectively. Labelling systems used were the streptavidin–biotinylated horseradish peroxidase complex (Amersham) for hCuZnSOD, and the avidin–biotin complex (Vectastain Elite kit, Vector) for the other proteins.

Double-labelling experiments were performed by processing the slides for βgal histochemistry, followed by TH- or OX42- immunohistochemistry.

Quantification of tyrosine hydroxylase-positive cells

The number of surviving TH-immunoreactive neurons in each graft was assessed by manual counting on every third section on blind-coded slides. The raw values were corrected according to the Abercrombie formula,[1] with the mean cell diameter estimated at 16.4 µm, 17.1 µm and 17.1 µm for the control, βgal and SOD groups, respectively.

Statistical analysis

A two-factor repeated measures analysis of variance (ANOVA) was used to determine differences in rotational asymmetry between groups over time. One-factor ANOVA with *post hoc* Scheffe's F-test was used for intergroup comparisons of rotation scores for single time-points, and paired t-test was used for intragroup comparisons over time. One factor ANOVA was used to compare numbers of surviving TH-immunoreactive neurons between groups.

RESULTS

Cell suspensions of embryonic VM tissue, either uninfected or infected with the βGal or SOD adenoviruses, were transplanted into the striatum of the denervated rats (eight rats/group). Two rats/group were perfused four days after transplantation for morphological analysis, and the other rats were tested for amphetamine-induced turning behaviour before sacrifice and histological examination.

Table 1. Amphetamine-induced rotation asymmetry

	Pregrafting	Two weeks postgrafting	Five weeks postgrafting
Control (n=6)	7.1 ± 0.7	10.1 ± 2.2	3.1 ± 2.7
βgal (n=6)	9.9 ± 2	9.2 ± 2.6	4.7 ± 2.1
SOD (n=6)	8.8 ± 1.6	6.5 ± 0.7	−6.3 ± 1.7

Net rotational asymmetry score (full turns contralateral to the lesion substracted from turns ipsilateral to the lesion/min).
Data are given as means ± SEM.

Rotational behaviour

The number of amphetamine-induced rotations ipsi- and contralateral to the lesion were monitored before transplantation, and two weeks and five weeks after transplantation. Net rotation asymmetry scores are summarized in Table 1. Prior to transplantation, the net ipsilateral rotation asymmetry was not different between the three groups (one factor ANOVA $F_{2,15}$=0.83, P>0.05). At two weeks after transplantation, the proportions of rats that exhibited at least 50% reduction in net motor asymmetry compared to pre-transplantation values were 0/6, 2/6, and 1/6, for the control, βgal and SOD groups, respectively. At five weeks post-transplantation, these proportions were 3/6, 4/6 and 6/6 (2/6 rats exhibited more contralateral turns than ipsilateral in each of the control or βgal groups, and this proportion was 5/6 for the SOD group). A two-factor repeated measures ANOVA revealed a significant difference for the net ipsilateral asymmetry between groups [$F_{2,45}$=5.44, P<0.01], within groups across time [$F_{2,45}$=18.1, P=0.0001], and between groups across time [group × time interaction, $F_{4,45}$=2.89, P<0.05]. At two weeks after implantation, the rotation score was not significantly different between groups [one-factor ANOVA $F_{2,15}$=0.88, P0.05]. At five weeks after surgery, there was a significant difference between groups for the net rotation asymmetry [one-factor ANOVA, $F_{2,15}$=7.54, P<0.01]: the rotation score was significantly reduced in the SOD group compared to the control and βgal groups (*post hoc* Scheffe's F-test, P<0.05 and P<0.01, respectively). Only the SOD group exhibited complete reversal of rotation asymmetry and displayed a mean score that was significantly reduced compared to pre-transplantation and two weeks post-transplantation values (paired Student's t-test, P<0.01).

Striatal expression of the transgenes

Human CuZnSOD cDNA and the *E. coli lacZ* gene were introduced into rat embryonic VM cells using the adenovirus infection method previously used for cell cultures and now modified for freshly-prepared cell suspensions. VM cells were infected at a MOI of 25 pfu/cell, a viral concentration that we have previously found to direct the production of

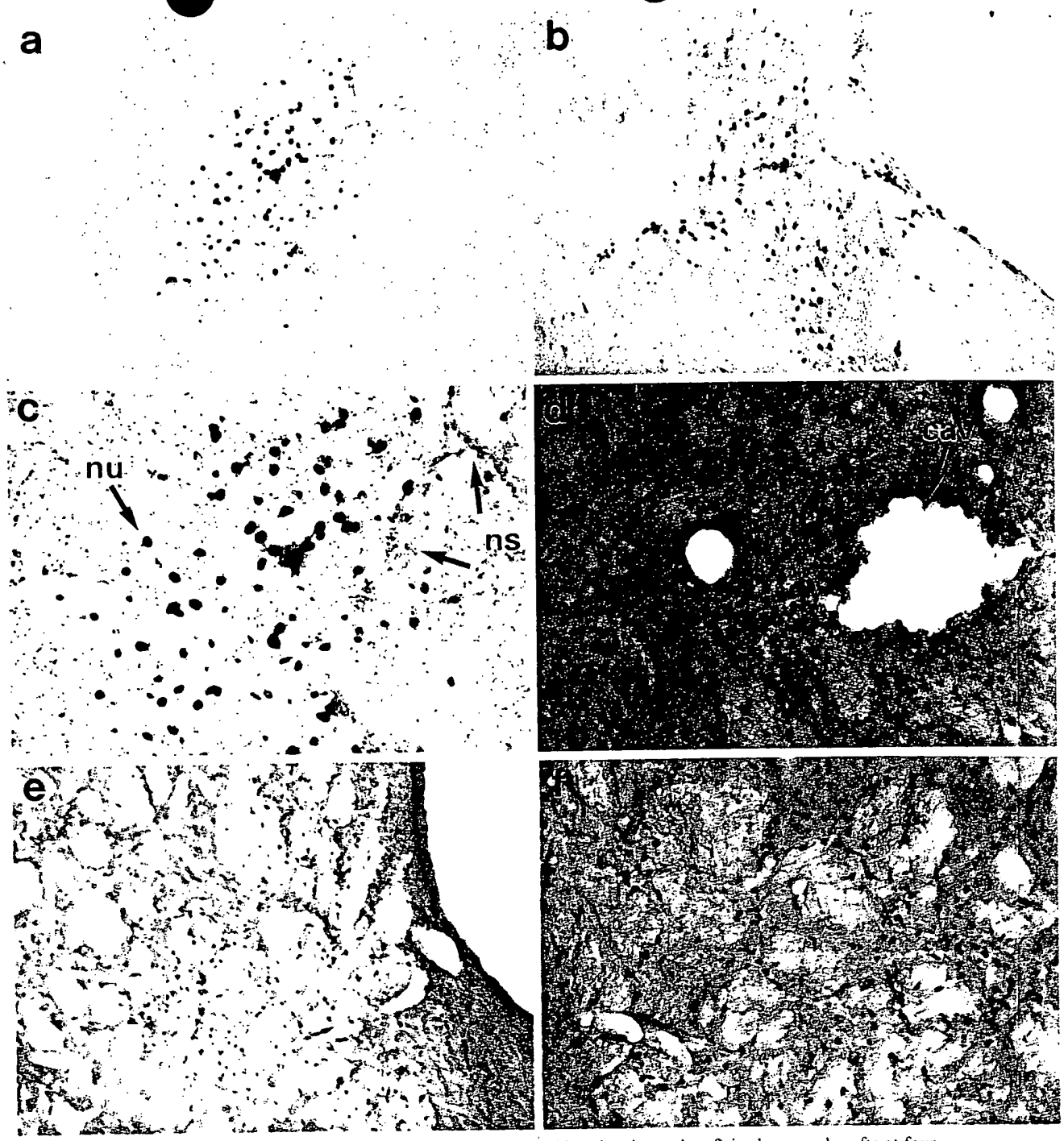

Fig. 1. X-gal histochemistry (a, b, c) and βgal immunohistochemistry (d, e, f) in the neural grafts at four days (a, c, d) and five weeks (b, e, f) after intra-striatal transplantation of embryonic mesencephalic tissue infected with the βgal adenovirus. a, b, d, e) low magnification of βgal-positive cells (scale bar=100 μm). An example of cavity (cav) is illustrated in d. c, f) high magnification of βgal-positive cells (scale bar=100 μm). Specific blue staining of βgal-positive nuclei (nu) and non-specific blue staining (ns) are illustrated in c.

hCuZnSOD or β-gal in cultured cells, without significant toxicity for TH-immunoreactive neurons (unpublished observations).

Expression of β-galactosidase. At four days after the graft surgery, two of the eight rats grafted with Ad-RSVβgal-infected cell suspensions were killed and showed surviving transplants (Fig. 1a,c,d). However, at this time-point, there were often cavities within the graft tissue in all groups (Fig. 1d). These cavities possibly represented areas where the graft tissue was not completely integrated into the host striatum. Using βgal histochemistry (X-gal staining), several blue coloured nuclei were found to be

clustered inside the graft and around the injection site (approximately 50–150 βgal-positive cells/graft section, Fig. 1a,c). These nuclei correspond to βgal-producing cells in which the *lacZ* gene expression was targeted to the nucleus by the SV40 nuclear localization signal.[45]

The expression of βgal was still detected at five weeks after transplantation in the six transplanted rats of the βgal group using X-gal histochemistry (Fig. 1b) (approximately 50–150 βgal-positive cells/graft section).

With the X-gal staining procedure, it is possible to detect non-*E. coli* lacZ staining in cells with neuronal, glial and endothelial morphology in the normal adult rat brain;[35] we thus performed an immunohistochemical procedure using an antibody specific for the *E. coli* βgal. A similar labelling pattern was obtained whether using X-gal histochemistry (Fig. 1a,b,c) or βgal immunohistochemistry (Fig. 1d,e,f) staining procedures, although some non specific X-gal labelling was sometimes observed around blood vessels (Fig. 1c). No non-specific βgal immunostaining was observed in brain sections from rats grafted with Ad-hCuZnSOD-infected cell suspension (not shown).

As βgal was targeted to the nucleus, only nuclei were stained and this rendered the morphological characterization of cells impossible. Thus, we could not discriminate the cell types that were infected with the RSVβgal adenovirus. Co-labelling experiments were performed on a few sections using X-gal histochemistry and TH or OX-42 immunochemistry. There were several cells labelled with either antibody or the X-gal histochemistry; however, in these sections, we did not find any *lacZ*-positive cells that were clearly double-labelled.

Expression of human Cu/Zn superoxide dismutase. In rats implanted with Ad-hCuZnSOD-infected cell suspension, we assessed the expression of hCuZnSOD in the grafts using an antibody which discriminates between endogenous rodent CuZnSOD and the exogenous human form of the enzyme.[3] At four days after surgery, two of the eight rats grafted with the hCuZnSOD adenovirus were killed and these contained surviving transplants with human CuZnSOD-immunoreactive cells (Fig. 2a,c). As in the βgal group, cavities were found within the graft tissue. The immunoreactive cells were uniformly stained for the hCuZnSOD recombinant protein throughout the cytoplasm and dendritic processes, which permitted the identification of positive cells having neuron-like or glia-like morphology (Fig. 2c) (with approximately 20–50 hCuZnSOD-positive cells/graft section).

Human CuZnSOD immunostaining was still detected at five weeks post-implantation and morphological examination of the cells indicated that a minority of infected cells had a clear neuronal phenotype (Fig. 2b,d). Most of the cells, located inside or around the graft, resembled microglia (Fig. 2d). In most grafts, the intensity of the hCuZnSOD immunostaining appeared slightly weaker at five weeks postgrafting when compared to postgrafting at four days.

We did not detect any cortical X-gal or CuZnSOD labelling consecutive to retrogradely-transported free virus along the corticostriatal pathway in rats grafted with either Ad-hCuZnSOD- or Ad-RSVβgal-infected cell suspensions (not shown).

OX-42 immunohistochemistry

The presence of an inflammatory response was evaluated by OX-42 immunohistochemistry (Fig. 3). This antibody detects rat complement receptor type 3, which is located on macrophages and microglia.[34] At four days after transplantation, there was more intense OX-42 staining in the rats grafted with infected cell suspensions (Fig. 3b,c) than in the control group (Fig. 3a). No obvious difference was detected between rats grafted with either Ad-hCuZnSOD- or Ad-RSVβgal-infected transplants.

The majority of immunoreactive cells were found within the graft tissue and had an activated microglia phenotype with an enlarged intensely-stained soma and several distinctly-labelled processes with multiple arborizations. Several round or cuboidal cells without processes, which resembled macrophages, were also found within and around the grafts. In the contralateral hemisphere, there were several weakly-stained cells with the characteristics of resting microglia cells.

The intensity of OX-42 staining decreased significantly between four days and five weeks after transplantation in both the βgal (Fig. 3b,e) and SOD (Fig. 3c,f) groups. At five weeks postgrafting, most of the immunoreactive cells had a microglia morphology and were located at the interface between graft and host tissue.

Graft survival

Light microscopic analysis of TH-immunostained brain sections (Fig. 4) revealed two surviving grafts in the striatum of each rat from the SOD and the control groups. In one of the SOD rats, the two grafts were inadvertently placed in the caudal striatum, close to the globus pallidus. One of six control rats had two grafts that were located in the lateral striatum. In the βgal group, 3/6 rats had two surviving grafts, 2/6 had one surviving lateral graft, and 1/6 did not have any surviving graft (this later animal was thus excluded from the statistical analysis).

TH-immunoreactive neurons in each graft were added for each rat and the mean number of surviving TH-positive neurons in each group was 443 ± 129, 347 ± 130, and 700 ± 150 for the control, βgal and SOD group, respectively (Fig. 4). There was no statistically significant difference between groups for the mean number of surviving TH-positive cells in the

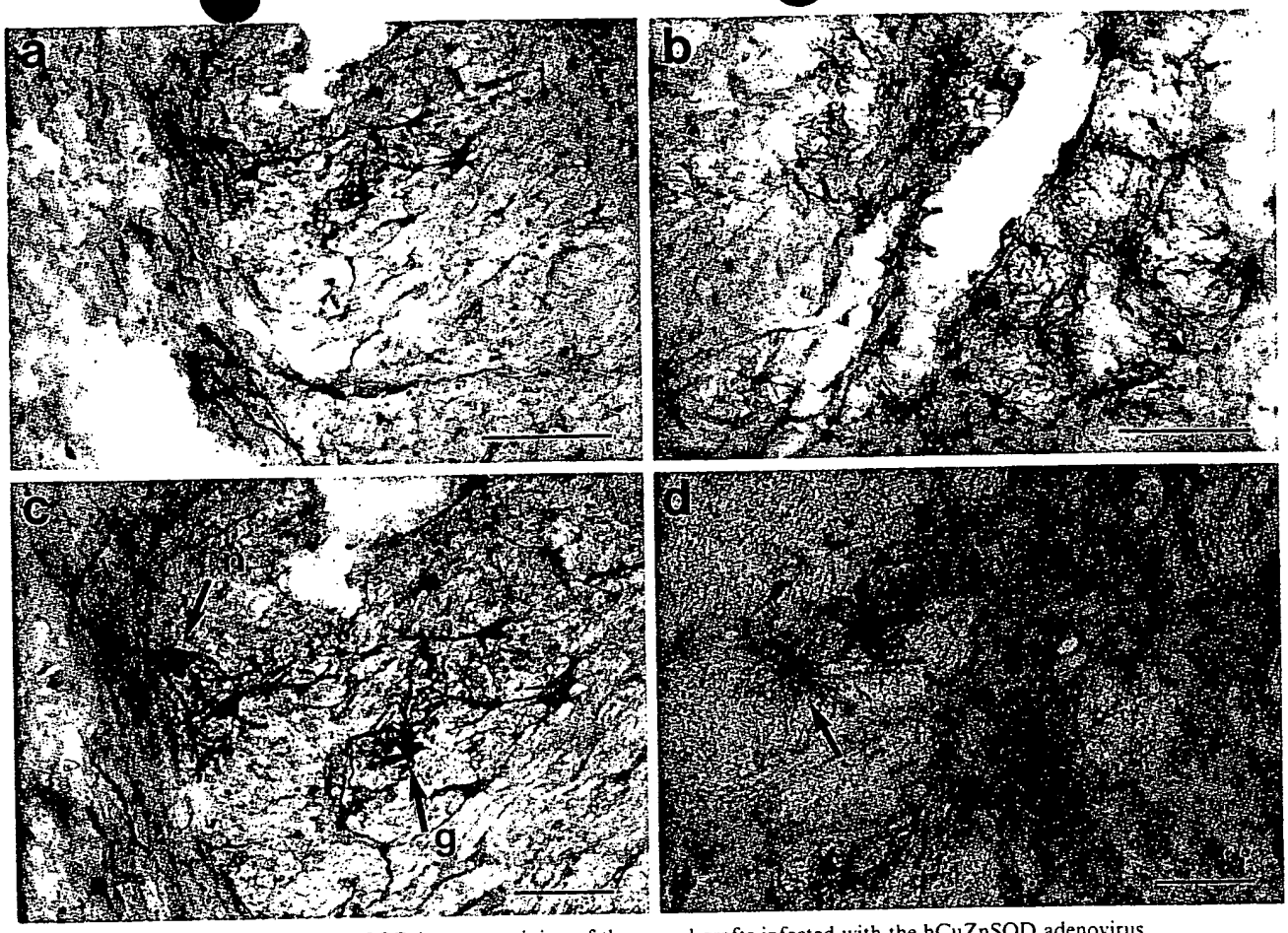

Fig. 2. Human CuZnSOD immunostaining of the neural grafts infected with the hCuZnSOD adenovirus analysed at four days (a, c) and five weeks (b, d) post-transplantation. a, b) Low magnification of hCuZnSOD-positive cells inside the graft (scale bar=100 μm). c) High magnification of hCuZnSOD-positive cells with a "neuron-like" (n) or "glia-like" (g) morphology (scale bar=30 μm). d) High magnification of hCuZnSOD-positive cells (scale bar=30 μm) showing a non-neuronal cell (arrow).

grafts (one-factor ANOVA, $P>0.05$), although a trend was observed with a higher TH cell number in the SOD group. Since approximately 160,000 cells were implanted in each animal, the mean yield of surviving TH-positive neurons in the three groups was approximately 3.1/1000 injected cells.

Correlation between graft survival and function

The sum of TH-positive cells found in each rat was plotted against the percent reduction in net rotation asymmetry at five weeks postgrafting. Logarithmic regression analysis showed a significant correlation between the two parameters ($r^2=0.510$, $P<0.01$). From the curve obtained by the analysis, we deduced that approximately 500 TH-positive neurons were necessary to induce a 50% recovery in rotational behaviour (unpublished observations).

DISCUSSION

Previous studies have shown that intrastriatal *in vivo* gene transfer using viral vectors encoding for the *TH* gene can result in significant behavioural recovery in 6-OHDA lesioned rats.[12,16,17] In these studies, viruses were directly injected into the denervated striatum. Thereby host striatal cells were genetically modified to produce dopamine (DA), most probably resulting in a reduction in striatal DA receptor supersensitivity which was manifested as an amelioration of apomorphine-induced motor asymmetry.[12,16,17] Alternatively, intrastriatal grafting of mesencephalic DA neurons can substitute for the lost DA innervation in the striatum of lesioned rats (for review see Ref. 4). Recent studies have demonstrated that in a rat model of PD, there was an improved survival of grafted dopamine neurons that had been treated with exogenous antioxidant[24] or taken from transgenic mice that overexpress hCuZnSOD.[25] Intracellular production of active hCuZnSOD protein in grafted neurons may be attained by using a replication-defective adenovirus vector for *ex vivo* transfer of the hCuZnSOD gene, i.e. *in vitro* gene transfer into cells that are subsequently transplanted into the brain. Such a method has been recently described for human neural progenitors infected *in vitro* by an adenovirus carrying the *E. coli lacZ* marker gene and then transplanted into the brain of

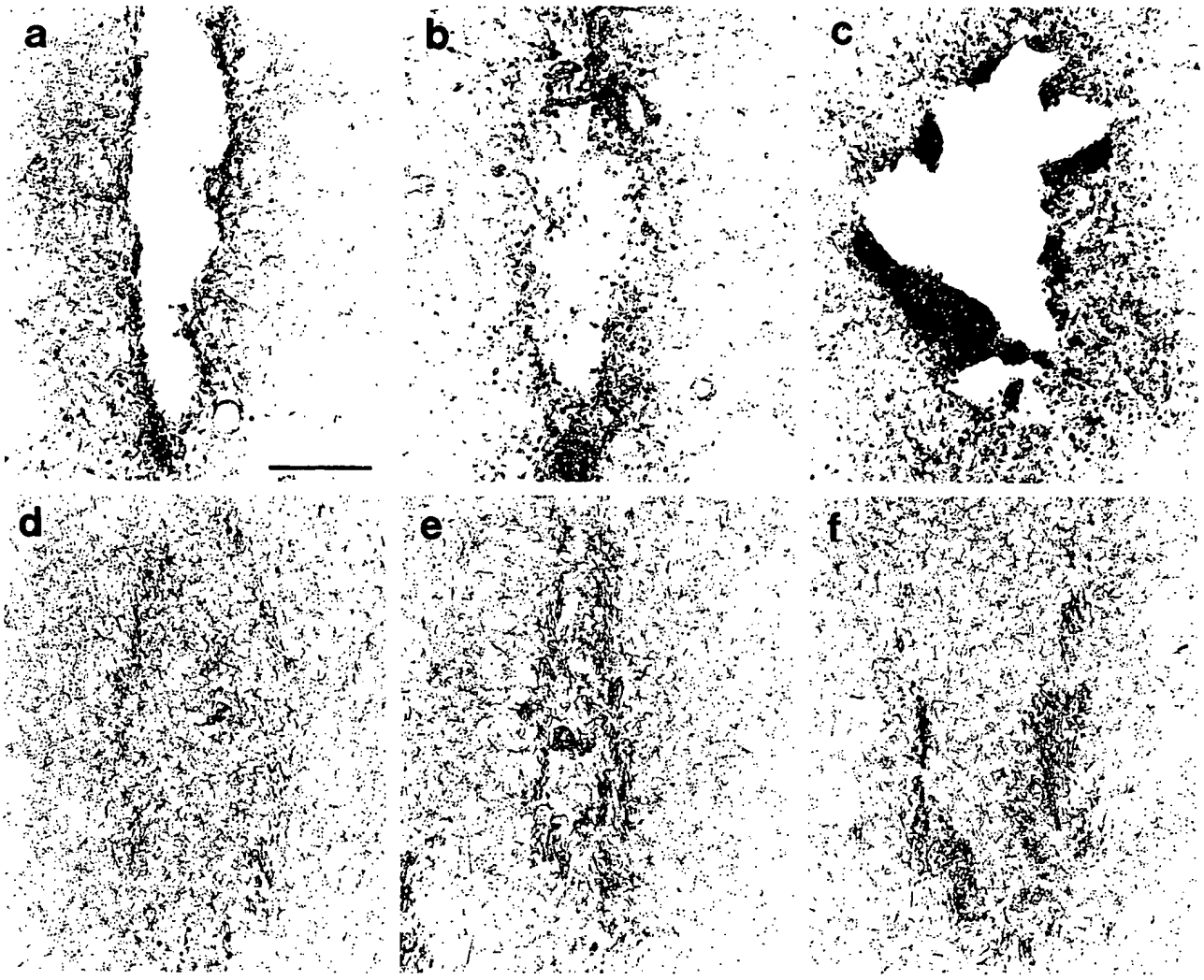

Fig. 3. Microglia/macrophage (CR3) immunostaining of coronal sections through the neural grafts at four days (a,b,c) and five weeks (d,e,f) after transplantation. a, d) Control; b, e) βgal; and c, f) SOD representative neural grafts (scale bar=250 μm).

adult rats.[38] The main objective of our present study was to examine whether embryonic VM tissue could be infected *in vitro* with an adenovirus and then grafted into the adult brain without a reduction in transplant survival or function. Our present results demonstrate that the hCuZnSOD and βgal proteins are expressed within and around intrastriatal grafts of embryonic dissociated mesencephalic tissue previously infected with adenoviruses bearing either the hCuZnSOD cDNA (Ad-CuZnSOD) or the *E. coli lacZ* gene (Ad-RSVβgal).

The adenovirus vector-driven expression of the exogenous proteins was seen at four days after transplantation and persisted for at least five weeks thereafter. Inflammatory consequences of the adenovirus gene transfer appeared minimal: only a moderate microglial response, detected using immunocytochemistry for complement receptor 3 (CR3), was observed around the graft tissue. This weak inflammatory response was probably induced by the virion particles rather than by the transgenes: direct intracerebral injection of replication-defective adenoviruses either containing or lacking the *E. coli lacZ* gene was previously reported to lead to a substantial inflammatory response mediated by the virion particles themselves.[9] At 30 days after injection, the authors reported that CR3 expression (labelling macrophages and microglia) did not differ between virus- and buffer-injected animals.[9] This is in agreement with our present observation of a moderate to intense CR3 expression in and around all grafts at four days after transplantation and a relatively low microglial response at five weeks after surgery. Furthermore, our results demontrate that adenoviral-infection of VM cell suspensions prior to intrastriatal implantation did not have detrimental effects on the survival of DA neurons in the graft. On the contrary, there was a trend for a better survival of TH-immunoreactive grafted cells derived from Ad-hCuZnSOD-infected cell suspensions. Our results differ from those in a recent study using a defective herpes simplex virus vector system to transfer *ex vivo*

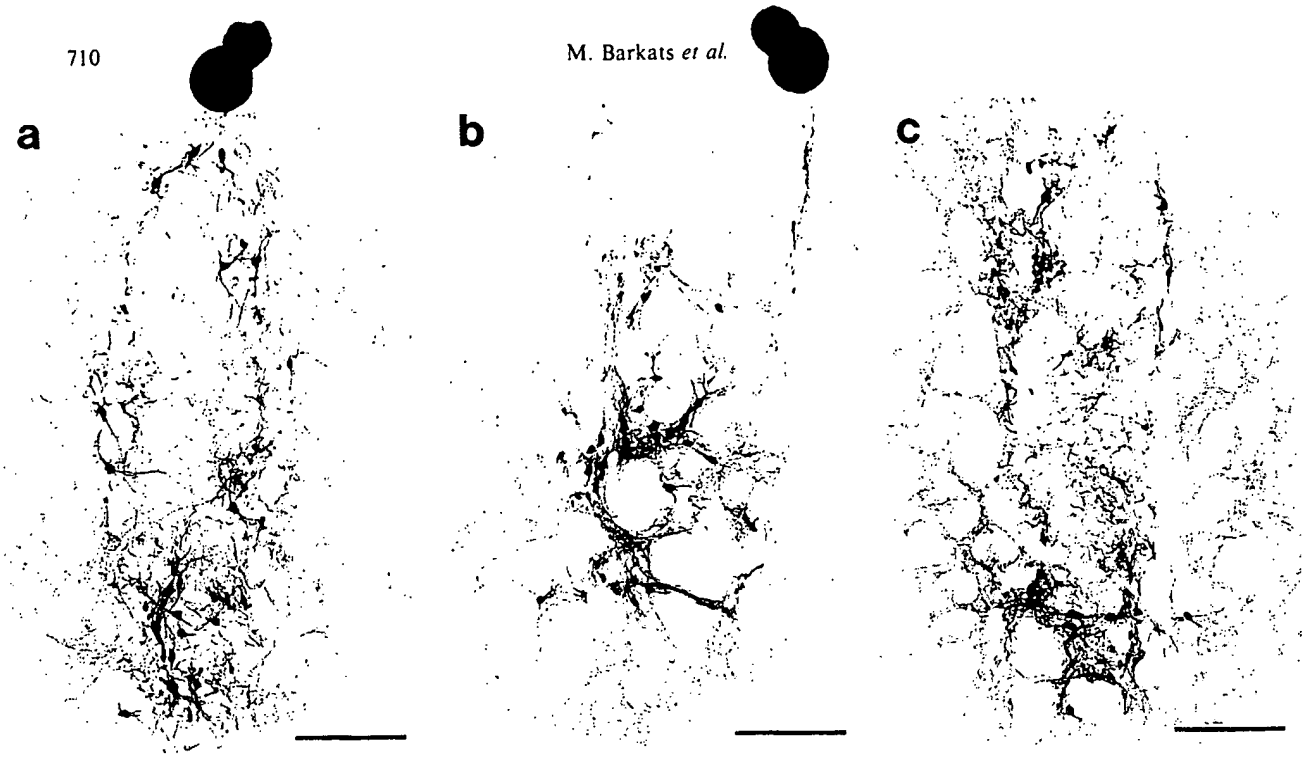

Fig. 4. TH-immunostaining of coronal sections through the neural grafts at five weeks after transplantation in one representative rat from each group. a) Control; b) βgal; and c) SOD groups are illustrated (scale bar=250 μm).

either the *lacZ* or the *TH* gene into the rat brain.[39] In this study, the authors infected neocortical cells prior to transplanting them into the rat striatum, but did not find a single TH-expressing cell in the grafts (they found only a few lacZ-positive cells). In another recent study using adenoviral vectors, addition of recombinant adenoviruses to mesencephalic tissue grafts reduced the survival and differentiation of TH-positive neurons in the transplants.[37] In this report, grafted cell suspensions had been supplied with 3×10^7 pfu of adenoviral vectors encoding either *E. coli* LacZ or human brain-derived neurotrophic factor. In our present study, the use of lower concentrations of recombinant adenovirus (4×10^6 total pfu) may explain why there was no impairment of graft survival and only a moderate inflammatory response in the transplants. Nevertheless, this low multiplicity of infection was sufficient to induce transgene expression in the brain grafts. Non-specific inflammation has been postulated to increase the risk of rejection of histoincompatible neural grafts by up-regulation of transplantation antigens.[49] This mechanism is not applicable to our present experiment, because the donor-host combination was syngeneic. Conceivably, expression of viral antigens on the surface of grafted cells may stimulate immune destruction of some infected cells.[2] However, our results suggest that there was no apparent intense immune rejection of adenovirus-infected cells.

The use of Ad-hCuZnSOD and Ad-RSVβgal for *ex vivo* gene transfer did not seem to inhibit the functional capacity of the grafted cells and their ability to reverse motor asymmetry in rats with experimental PD. In fact, the onset of functional transplant effects in rats transplanted with Ad-hCuZnSOD-infected cell suspensions was faster than in those receiving untreated control tissue or grafts infected with Ad-RSVβgal. The significantly faster and more extensive functional recovery observed in the SOD group was not reflected in a statistically significant increase in DA graft cell number in this group, although there was a strong trend for enhanced graft survival. The number of surviving DA neurons per grafted embryonic VM (each host received the equivalent of 0.6 embryonic VM) was 700–1200, which is in the lower range of previously reported results.[22,24,26,43] The relatively poor survival rate in all groups may be related to the fact that in this experiment the tissue was vigorously dissociated into single-cell suspensions, rather than maintained as a mixture of single cells and small aggregates.[7] There is strong evidence from several earlier studies[6,24,25,41,43] that the number of surviving DA neurons in a graft governs the extent of recovery in the amphetamine-induced rotation test. In our present study, there was also a significant correlation between the logarithm of the number of TH-positive cells and the percentage reduction in rotation asymmetry. It may thus seem surprising that the SOD group, which displayed greater functional recovery, did not carry significantly larger grafts. However, recovery of rotational behaviour is also related to precise graft placement[11] and extent of fibre outgrowth.[5] Small variations in these parameters, combined with the strong trend towards larger grafts could have resulted in the observed enhanced functional effects.

The trend for an improved graft survival in the SOD group, compared to the βgal and control group, could be associated with the potential neuroprotective effect of intracellular overexpression of SOD. This enzyme plays a crucial role in the free radical detoxification by scavenging the superoxide anion (O_2^-) which has been shown to be neurotoxic either directly or through its reaction with nitric oxide.[10,18,47] Oxidative stress potentially generated by cellular hypoxia and trauma that occur during preparation and implantation of embryonic mesencephalic tissue could therefore be reduced by intracellular overexpression of SOD in grafted neurons. Possibly, SOD expressed in non-neuronal cells may also indirectly enhance the functional recovery of the grafts by increasing the survival and/or function of astrocytes stimulated in the graft or in the host tissue: glial cells are known to play an important role in the survival of neurons by secreting growth factors (such as glial-derived neurotrophic factor[20]) or by providing cysteine which is taken up by neurons for glutathione synthesis.[40] Reactive oxygen species are potentially generated by activated phagocytes (e.g., brain macrophages and microglia):[23] these possess an enzymatic complex, the NADPH oxidase, which catalyses the formation of superoxide upon exposure of phagocytes to appropriate stimuli (infection, grafting...). A defective phagocyte superoxide production has been reported to alter the inflammatory response in a mouse model of chronic granulomatous disease.[32] In rats grafted with the Ad-hCuZnSOD-infected cell suspensions, the over-expression of SOD in infected phagocytes may lead to defective immune response mechanisms linked to superoxide generation. This could explain the tendency of an enhanced graft survival and the significant behavioural recovery in the SOD group. It is well known that free radical species can diffuse over membranes and that by-products of lipid hydroperoxides can damage the membranes of neighbouring cells[30] and quenching of these by non-neuronal cells could also be relevant for protection of the DA cells (by disruption of chain reactions of peroxidation).

In our study, all grafted rats had surviving transplants, and if infected, all of the transplants expressed the βgal or hCuZnSOD transgenes. A differential expression of the hCuZnSOD and βgal proteins was found in the grafts (number of βgal-immunoreactive cells hCuZnSOD-immunoreactive cells), which may be due to possible differences in the sensitivity of the two immunostaining procedures, or the stability of the two proteins.

The number of the *ex vivo* infected cells that expressed the exogenous proteins appeared lower in our study than in the report described by Sabaté *et al.*[38] However, this may be explained by one or more of the differences in the experimental protocols between the two studies. For example, ten times fewer cells were injected in our present study (150,000 cells/rat instead of 1,000,000), a lower viral concentration was employed (25 pfu instead of 500 pfu), the incubation time in the viral solution was reduced (1 h instead of overnight) and we performed the *ex vivo* infection in a cell suspension instead of in culture. The changes in most of these experimental conditions were necessary to optimize conditions for the survival of the DA neurons.

At four days after the graft surgery, cells expressing the transgenes had a neuronal and/or a glial phenotype. However, at five weeks post-transplantation, most transgene-expressing cells resembled microglia, although sporadic cells with neuronal morphology were found in the grafts. That may reflect a higher down-regulation of the transgene expression in neurons than in microglia, or differences in sensitivity of neurons and microglia to the immune reaction triggered by the viral infection. Unlike at four days postgrafting, many labelled cells were found out of the grafts at five weeks postgrafting (Fig. 1b,e), which may correspond to their migration away from the implantation site to populate a larger striatal area.[27]

Double-labelling experiments failed to show a co-localization of the βgal transgene and the TH. However, previous *in vitro* experiments showed that approximately 5–10% of the TH-infected VM cells were co-labelled with βgal (data not shown). *In vivo* experiments of direct Ad-RSVβGal injection into the substantia nigra also previously showed that about 50% of βgal-positive cells in the substantia nigra co-expressed TH, thereby demonstrating the potentiality of DAergic neurons to be infected with adenoviruses.[19] As few TH cells were found in the sections that we used for the double-labelling experiment (10–20 TH-positive cells), it is not surprising that none of them were clearly βgal-positive. Moreover, our double-staining analysis was carried out at five weeks after transplantation, and with regard to not finding double-labelled TH cells at this time-point does not exclude the possibility that such TH cells expressed the transgenes at the moment of the grafts. Previous *in vitro* experiments where the embryonic mesencephalic rat cells were infected with the SOD adenovirus provided double-immunostaining evidence that at least 5–10% of the TH cells clearly expressed exogenous hCuZnSOD a few days after infection (unpublished observations). Oxidative stress is potentially generated by cellular hypoxia and trauma that occur during preparation and implantation of embryonic tissue and could have deleterious effects only during the first hours and days after transplantation. In this context, only the early expression of SOD within the cells should be essential for neuroprotection. Furthermore, a down-regulation of transgene expression has already been observed in transplanted cells genetically modified using retroviruses, and could be a result of gene regulation when using viral promoters.[31] In our present study, such a suppression of the transgene expression (under the control of the RSV promoter)

could explain the failure to find clearly double-labelled TH neurons at five weeks postgrafting.

Possibly, host striatal cells could be infected by free adenoviruses remaining in the supernatant of the grafted cell suspensions. However, after 1 h incubation at 37°C, most of the free virions should have been inactivated. If there had been free virions present, probably some of them would have been taken up by nerve terminals at the injection site and transported retrogradely[9,13,33] e.g., along the corticostriatal pathway. Thus, the absence of cells exhibiting labelling for βgal or CuZnSOD in the cortex of the rats in our present study suggests that there were not significant amounts of free virions in the cell suspension supernatants.

CONCLUSION

Here, we described a promising *ex vivo* gene transfer method using a replication-defective adenovirus bearing the human CuZnSOD gene, which could be used in attempts to enhance functional efficacy and maybe survival of grafted DA neurons. Further studies will be essentially directed towards the improvement of the infection efficiency, to obtain a high level transgene expression with a minimal inflammatory reaction.

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